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トランスクリプトソーム解析による医薬品の副作用機構の解明と、その副作用感受性診断、及び創薬への応用

平成 20 年度　総括研究報告書

主任研究者　水島　徹

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主任研究者　水島　徹　熊本大学大学院医学薬学研究部教授

研究要旨

我々は、我々が確立した薬剤性間質性肺炎に関する動物モデルにおいて、薬剤性間質性肺炎を起こす種々の薬剤効果を検討し、全ての薬剤が間質性肺炎様症状を起こすことを見出した。また PC-SOD やピルフェニドンにより、この症状が改善することを見出した。一方我々は、中用量ブレオマイシンをあらかじめ投与したマウスにレフルノミドを投与すると、TNF-αを投与しなくても間質性肺炎様症状が誘導されることを見出した。またこの時、上皮細胞の上皮間葉転換が起きることを見出した。以上の結果は、レフルノミドは上皮間葉転換を起こすことにより、薬剤性間質性肺炎を誘導していることを示唆している。

A. 研究目的

医薬品の副作用、特に副作用感受性に関する個人差が臨床現場で大きな問題になっている。問題は、その副作用の発症機構が充分に理解されていないため、新薬候補品の副作用、及び患者の副作用感受性を予測出来ない点である。そこで本研究で我々はトランスクリプトソーム解析を用いて医薬品の副作用発症機構を解明し、新薬候補品の副作用、及び副作用感受性に関する個人差を予測する方法を確立する。この研究は以下に述べるように、新薬の開発にも繋がる。以下に我々がこれまで行ってきた、非ステロイド系抗炎症薬（NSAID）に関する研究の成果を述べる。

アスピリンを代表とする NSAID は優れた抗炎症薬として世界中でよく使用されているが、その胃潰瘍副作用（NSAID 潰瘍）が臨床現場で大きな問題になっている（米国では年間 16500 人が NSAID 潰瘍で亡くなっている）。我々は NSAID が誘導する遺伝子をストレス遺伝子チップ（自ら開発した、ストレス遺伝子に特化した DNA チップ）で解析し、NSAID が胃粘膜細胞死を誘導すること、及びこの細胞死が NSAID 潰瘍の原因であることを見出した。実際、市販されている NSAID の細胞傷害性と胃潰瘍副作用の間には有意な相関性が見られた（副作用予測システムの確立）。この結果は、細胞傷害性の少ない NSAID は胃潰瘍副作用の少ない NSAID になることを示している。実際我々はそのような NSAID の合成に成功し、それらが充分な抗炎症作用を示すにも関わらず、ほとんど胃潰瘍を起こさないことを見いだした（現在前臨床試験中）。またこの細胞傷害に影響を及ぼす複数の遺伝子を同定し、その遺伝子多型により細胞の NSAID 感受性が変化することを見出したので、本研究で我々はこの成果を基に、患者の NSAID 潰瘍感受性を予測する方法を確立する。また最近我々は、臨床現場で間質性肺炎副作用が問題になっている抗癌剤や抗リウマチ薬に関しても、ストレス遺伝子チップによるトランスクリプトソーム解析を行った。その結果、これらの医薬品が抗炎症作用を持つタンパク質の発現を強く抑えることを見出した。またこれまで成功していなかった薬剤性間質性肺炎の実験動物モデルの確立に成功し、これらの抗炎症タンパク質の減少が薬剤性間質性肺炎の原因になっていることを示唆した。以上の成果を受けて本研究で我々は、薬剤性間質性肺炎、及び他の医薬品副作用に関して、その発症機構を解明し、新薬候補品の副作用、及び患者の副作用感受性を予測する方法を確立すると共に、副作用の少ない新薬の開発に向けた研究も行う。

B. 研究方法

抗癌剤（ゲフィチニブ（イレッサ）な

ど)、抗リウマチ薬(レフルノミド、エタネルセプトなど)、漢方薬(小紫胡湯など)による間質性肺炎副作用(薬剤性間質性肺炎)が臨床現場で大きな問題になっているが、その発症メカニズムはほとんど分かっていない。また欧米では、我が国ほど薬剤性間質性肺炎は問題になっていない。そこで薬剤性間質性肺炎発症機構を解明し、新薬候補品の副作用、及び患者の副作用感受性を予測する方法を確立すると共に、副作用の少ない新薬を開発することは大変重要である。これまでに我々は、これらの医薬品がSOD、HO-1、Nrf2、HSPなど抗炎症作用を持つタンパク質の発現を強く抑えることを見出している。

薬剤性間質性肺炎研究が遅れていたのは、その動物モデルが確立されていなかったためである。我々は、TNF-α(薬剤性間質性肺炎において重要な役割を果たしている)、及び低用量ブレオマイシン(高用量ブレオマイシン単独で、間質性肺炎症状が現れる)をあらかじめ投与したマウスに、レフルノミドやエタネルセプトを投与すると、間質性肺炎症状が現れることを見出し、薬剤性間質性肺炎モデルを確立したと考えている。このモデルにおいて、PC-SOD(SODを修飾し安定性を高めた製剤で、現在、間質性肺炎治療薬としての臨床試験中)、及びHO-1の誘導剤により、この間質性肺炎様症状が改善することを見出した。以上の結果は、これらの医薬品が抗炎症タンパク質を低下させることにより、間質性肺炎を引き起こしている可能性を示している。

そこで我々は、他の薬剤性間質性肺炎を起こす薬剤、及びその他の薬剤をこのモデルで検討し、このモデルが新薬候補品の間質性肺炎副作用を予測するシステムとして使用出来るかを検討する。また、SOD、Nrf2、HSPのノックアウトマウスや過剰発現マウスにおける薬剤性間質性肺炎を調べることにより、これらの因子が薬剤性間質性肺炎に関与していることを証明する。

C. 研究結果

今年度我々は、我々が確立した薬剤性間質性肺炎に関する動物モデル(TNF-α、及び低用量ブレオマイシンを投与したマウスにさらに薬剤を投与し、間質性肺炎様症状が誘導されるかを調べる)において、薬剤性間質性肺炎を起こす種々の薬剤(ゲフィチニブ、イマチニブ、パクリタキセル、アミオダロン、エタネルセプト、インフリキシマブ)の効果を検討し、全ての薬剤が間質性肺炎様症状(組織傷害、炎症性細胞の浸潤、組織の繊維化、肺機能の低下)を起こすことを見出した。またPC-SODやピルフェニドン(臨床試験において、間質性肺炎に対して有効性を示した医薬品)により、この症状が改善することを見出した。以上の結果は、

このモデルが新薬候補品の間質性肺炎副作用を予測するシステム、及び間質性肺炎治療薬の評価システムとして有用であることを示している。

一方我々はゲフィチニブなどが抗炎症タンパク質（SOD、Nrf2、HSP）の発現を強く抑えることを見出していた。そこで本年度我々は、これらタンパク質のノックアウトマウスにおける薬剤性間質性肺炎（上記のモデル）を調べ、これらのノックアウトマウスが薬剤性間質性肺炎を起こしやすいことを見出した。以上の結果から、ゲフィチニブなどはこれら抗炎症タンパク質を低下させることにより薬剤性間質性肺炎を起こしていることが考えられる。

一方我々は、中用量ブレオマイシンをあらかじめ投与したマウスにレフルノミドを投与すると、TNF-αを投与しなくても間質性肺炎様症状が誘導されることを見出した（より簡便な動物モデルの確立）。またこの時、上皮細胞の上皮間葉転換（EMT、最近間質性肺炎に深く関与していることが報告）が起きることを見出した。以上の結果は、レフルノミドはEMTを起こすことにより、薬剤性間質性肺炎を誘導していることを示唆している。

D.考察

結果の欄に記載した。

E.結論

このように平成20年度の我々の研究により、これまでほとんど分かっていなかった薬剤性間質性肺炎誘導機構がかなり明らかになり、また我々の確立した動物モデルが有用であることが示唆された。

F.健康危険情報

該当なし

G.研究発表

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H.知的財産権の出願・登録状況

1.特許取得

該当なし

2.実用新案登録

該当なし

3.その他

該当なし

研究成果に刊行に関する一覧表

雑誌

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Immunopharmacology and Inflammation

Inhibition of both COX-1 and COX-2 and resulting decrease in the level of prostaglandins E_2 is responsible for non-steroidal anti-inflammatory drug (NSAID)-dependent exacerbation of colitis ☆

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ABSTRACT

A number of clinical studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) exacerbate inflammatory bowel disease; however the molecular mechanism whereby this occurs remains unclear. NSAIDs inhibit cyclooxygenase (COX), which has subtypes COX-1 and COX-2. In this study, we have examined the effect of various types of NSAIDs on the development of dextran sulfate sodium (DSS)-induced colitis, an animal model of inflammatory bowel disease. The DSS-induced colitis was worsened by administration of non-selective NSAIDs but not by COX-1 or COX-2 selective inhibitors. However, administration of a combination of both COX-1- and COX-2-selective inhibitors exacerbated the colitis. The intestinal level of PGE_2 dramatically decreased in response to administration of COX-1- and COX-2-selective inhibitors, and exogenously administered PGE_2 suppressed the exacerbation of colitis by NSAIDs. The expression of mucin proteins, which protect the intestinal mucosa, was suppressed by non-selective NSAIDs and this expression was restored by PGE_2, both *in vivo* and *in vitro*. Intestinal mucosal cell growth was inhibited by non-selective NSAIDs and this cell growth was restored by PGE_2, both *in vivo* and *in vitro*. This study provides evidence that inhibition of both COX-1 and COX-2 and the resulting dramatic decrease in the intestinal level of PGE_2 is responsible for NSAID-dependent exacerbation of DSS-induced colitis. Furthermore, expression of mucin proteins and intestinal mucosal cell growth seems to be involved in this exacerbation and its suppression by PGE_2.

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1. Introduction

Inflammatory bowel disease has become substantial health problems. Recent studies suggest that inflammatory bowel disease involves chronic inflammatory disorders in the intestine due to the infiltration of activated leukocytes into intestinal tissues, the subsequent intestinal mucosal damage induced by reactive oxygen species that are released from the activated leukocytes, and, as a result of this mucosal damage, invasion of intestinal pathogenic bacteria across the intestinal mucosa (Podolsky, 2002). To develop a clinical protocol for the treatment of inflammatory bowel disease and to avoid accidental exacerbation of inflammatory bowel disease by clinically used drugs, it is important to know what type of drugs ameliorate or exacerbate the development of inflammatory bowel disease and to understand the underlying molecular mechanism. For this purpose, experimental animal colitis models, in particular the dextran sulfate sodium (DSS)-induced colitis models, are useful (Jurjus et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics and the anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins, which have a strong capacity to induce inflammation. Therefore, it was tentatively proposed that NSAIDs would be effective for the treatment of inflammatory bowel disease; however, clinical and animal studies have shown that NSAIDs exacerbate the development of inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Evans et al., 1997; Felder et al., 2000; Kabashima et al., 2002; Yamada et al., 1993). This seems to be due to the protective effects of prostaglandins on the intestinal mucosa through various mechanisms such as stimulation of mucin production, stimulation of mucosal cell growth, inhibition of mucosal apoptosis and inhibition of the production of pro-inflammatory cytokines (Kabashima et al., 2002). Supporting this notion, it was reported that inflammatory bowel disease-related experimental colitis can be attenuated by pre-treatment with exogenous prostaglandins (Tessner et al., 1998). However, it is not clear that NSAIDs exacerbate inflammatory bowel disease through decreasing the intestinal level of prostaglandins, because recent studies on gastric mucosa have shown that NSAIDs directly, in other words in a manner

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independent of COX-inhibition, damage gastric mucosal cells, which contributes to the formation of NSAID-induced gastric lesions (Tomisato et al., 2004). Furthermore, some studies have shown contradictory results which indicate that NSAIDs are safe for the treatment of inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Bonner et al., 2000; Takeuchi et al., 2006).

COX has two subtypes, COX-1 and COX-2. COX-1 is constitutively expressed in various tissues and play an important role in physiological homeostasis, whereas COX-2 is induced under inflammatory conditions, including in the intestine of inflammatory bowel disease patients and animal models of inflammatory bowel disease (Fukata et al., 2006; Singer et al., 1998; Tessner et al., 1998). Recently, COX-2-selective inhibitors have been developed as NSAIDs that are safer for gastric mucosa. Clinical and animal studies suggest that COX-2-selective inhibitors are also safe and, in some cases, beneficial for the treatment of inflammatory bowel disease patients and inflammatory bowel disease-related experimental colitis (El Miedany et al., 2006; El-Medany et al., 2005; Mahadevan et al., 2002; Martin et al., 2005); however, the mechanism by which they act is unclear. Furthermore, some studies have shown conflicting results: COX-2-selective inhibitors exacerbated the development of inflammatory bowel disease and colitis in animal models of inflammatory bowel disease (Bonner, 2001; Okayama et al., 2007; Reuter et al., 1996). On the other hand, the involvement of COX-1 in inflammatory bowel disease remains unclear. Since it is not uncommon for NSAIDs to be administered to inflammatory bowel disease patients accidentally or intensively (Evans et al., 1997), it is important to know what types of NSAIDs (for example, COX-1- and COX-2-selective inhibitors) are safe for inflammatory bowel disease patients. In this study, we have examined the effects of various NSAIDs on the development of DSS-induced colitis and found that the colitis was exacerbated by treatment with both COX-1- and COX-2-selective inhibitors. We also suggest that this exacerbation is due to a decrease in the intestinal level of PGE_2. Furthermore, we suggest that the protective effect of PGE_2 against DSS-induced colitis is mediated by various mechanisms, such as by induction of the expression of mucin proteins, stimulation of epithelial cell proliferation and suppression of reactive oxygen species-induced cell death in the intestine.

Table 1
Effect of various NSAIDs on colon shortening associated with DSS-induced colitis

Treatment	Length of colon (cm)
Vehicle	8.6±0.06
DSS	6.9±0.25
+SC-560 (2.5 mg/kg)	6.5±0.19
+SC-560 (5 mg/kg)	6.4±0.28
+SC-560 (10 mg/kg)	6.9±0.35
+Celecoxib (5 mg/kg)	6.9±0.25
+Celecoxib (10 mg/kg)	6.8±0.24
+Celecoxib (20 mg/kg)	7.2±0.28
+SC-560 (5 mg/kg) and Celecoxib (10 mg/kg)	5.8±0.24[a]
+Indomethacin (1 mg/kg)	5.8±0.06[a]

ICR mice were treated with or without 3% DSS for 7 days.
The indicated dose of each NSAID was administered daily. After 7 days, the colon lengths were determined as described in the Materials and methods. Values are mean±S.E.M. (n=4–15).
[a] $P<0.05$, vs. Control.

2. Materials and methods

2.1. Chemicals and animals

Celecoxib was from LKT Laboratories (St. Paul, MN). Paraformaldehyde, peroxidase standard, fetal bovine serum (FBS), o-dianisidine, 5-bromo-2′-deoxyuridine (BrdU), 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), menadione, hexadecyl trimethyl ammonium bromide (HTAB) and 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride (Hoechst 33342) were obtained from Sigma (St. Louis, MO). The antibody to BrdU was from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Indomethacin and DSS (M.W. 5000, 15–20% sulfur content) were from WAKO Pure Chemicals (Tokyo, Japan). Lipopolysaccharide (LPS) was

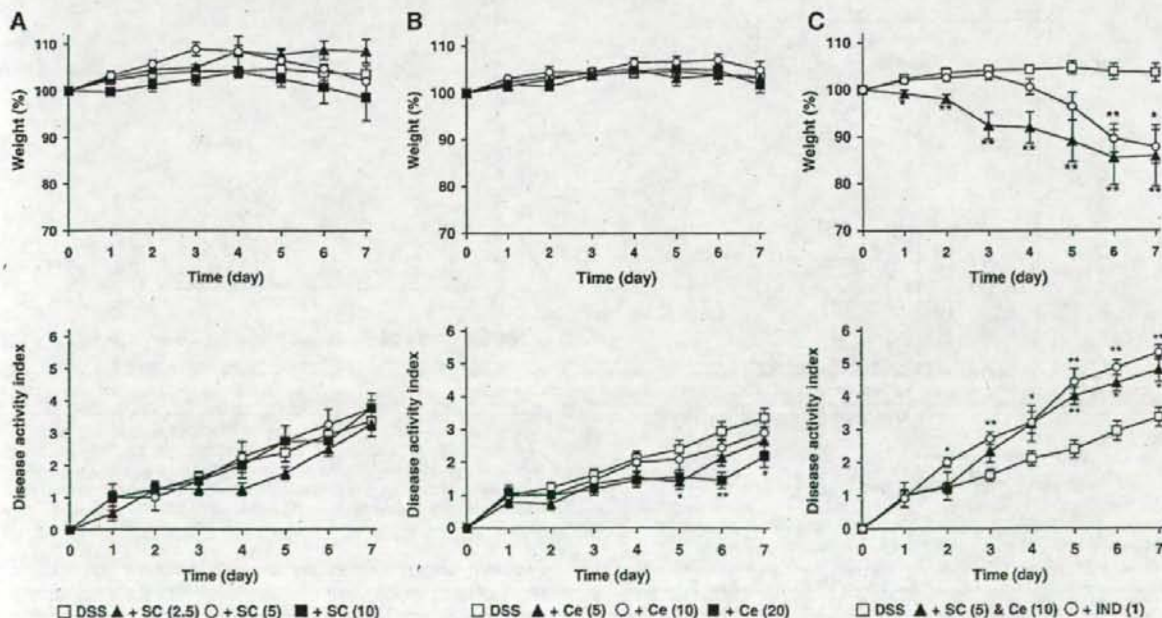

Fig. 1. Effect of various NSAIDs on DSS-induced colitis. DSS-induced colitis was developed as described in the legend of Table 1. The indicated dose (mg/kg) of SC-560 (SC) (A, C), celecoxib (Ce) (B, C) or indomethacin (IND) (C) was administered daily. Body weight and disease activity index were measured daily. Values are mean±S.E.M. (n=4–18). **$P<0.01$; *$P<0.05$.

from List Biological Laboratories, Inc (Campbell, CA). Optimal cutting temperature (O.C.T.) compound was from Sakura Finetek Japan (Tokyo, Japan). Mayer's hematoxylin and malinol were from MUTO Pure Chemicals (Tokyo, Japan). Terminal deoxynucleotidyl transferase (TdTase) was obtained from TOYOBO (Osaka, Japan). Alcian blue was from Nacalei Tesque (Kyoto, Japan). Nuclear fast red was from Merck KGaA (Darmstadt, Germany). The Envision kit was from Dako Co (Carpinteria, CA). Biotin 14-ATP and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories (Burlingame, CA). 4′, 6-diamidino-2-phenylindole-, dihydrochloride (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The RNeasy kit was obtained from Qiagen (Valencia, CA), first-strand cDNA synthesis kit was from GE Healthcare (Little Chalfont, UK) and iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560), the ELISA kit for PGE_2 and 16, 16-dimethyl PGE_2 (dm-PGE_2) were from Cayman Chemicals (An Arbor, MI). IEC6 (rat intestinal epithelial cell) and RAW264 (mouse leukemic monocyte) cells were from RIKEN BioResource Center (Tsukuba, Japan) and bEnd.3 (mouse

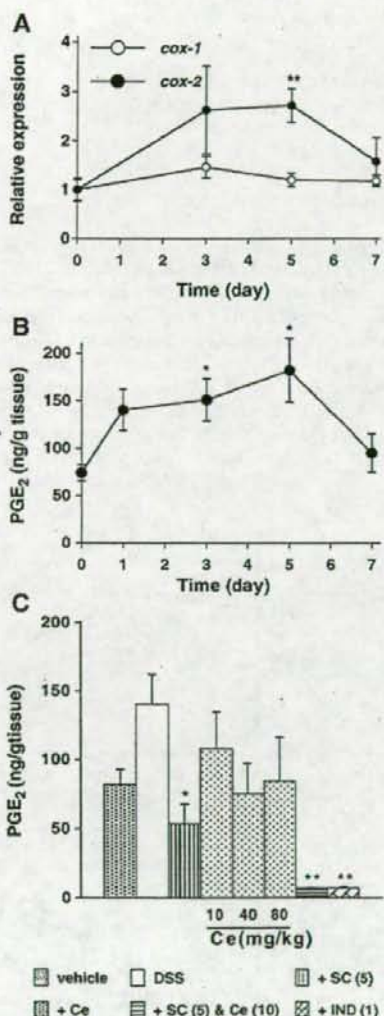

Fig. 2. The intestinal level of PGE_2 in DSS-treated mice. DSS-induced colitis was developed (A–C) and NSAIDs were administered (C) as described in the legend of Fig. 1. Colonic tissues were removed periodically and total RNA was extracted. Samples were subjected to real-time RT-PCR, using a specific primer set for *cox-1* or *cox-2*. Values were normalized to the *GAPDH* gene, expressed relative to the control sample (i.e. mice without DSS-treatment) (A). After the indicated number of days (B) or 3 days (C), colonic tissues were removed and PGE_2 levels were determined as described in the Materials and methods (B, C). Values are mean ± S.E.M. (n = 4–18). **P<0.01; *P<0.05.

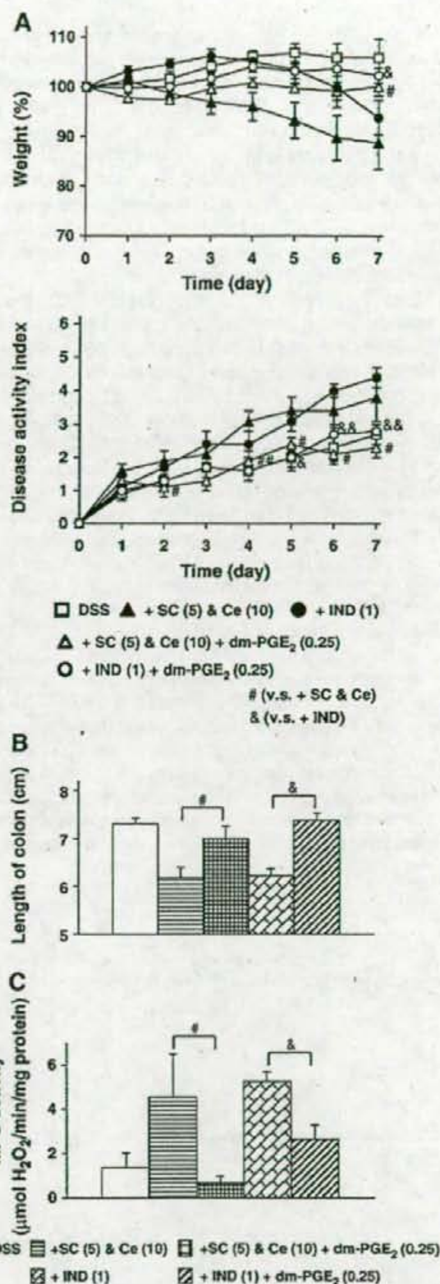

Fig. 3. Effect of dm-PGE_2 on exacerbation of DSS-induced colitis by NSAIDs. DSS-induced colitis was developed and NSAIDs were administered as described in the legend of Fig. 1. dm-PGE_2 (0.25 mg/kg) was administered twice per day. Body weight and (A) and colon length (B) were measured as described in the legend of Fig. 1 and Table 1, respectively. MPO activity was measured as described in the Materials and methods (C). Values are mean ± S.E.M. (n = 4–11). ## (or &&)P<0.01; # (or &)P<0.05.

brain endothelioma) cells were from the American Type Culture Collection (Rockville, MD). We used IEC6 cells because it is normal cell line and RAW264 and bEnd.3 cells because they are standard cell lines and used in many previous paper. ICR mice were from Kyudo Co. (Kumamoto, Japan). The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, and were approved by the Animal Care Committee of Kumamoto University.

2.2. Development of DSS-induced colitis and measurement of colon length and disease activity index

Colitis was induced in mice by the addition of 3% DSS (w/v, final concentration) to their drinking water as described previously (Tanaka et al., 2007). The animals were allowed free access to the DSS-containing water for 7 days. Over that time, NSAIDs were orally administered daily and dm-PGE_2 was intraperitoneally administered twice per day.

After 7 days, animals were placed under deep ether anaesthesia and sacrificed, the colons were dissected and their length measured from the ileocecal junction to the anal verge.

The disease activity index was determined macroscopically by an observer unaware of the treatment they had received, according to previously reported criteria (Tanaka et al., 2007). Briefly, the disease activity index was calculated as the sum of the diarrheal stool score (0: normal stool; 1: mildly soft stool; 2: very soft stool; 3: watery stool) and the bloody stool score (0: normal color stool; 1: brown color stool; 2: reddish color stool; 3: bloody stool).

For histopathological observation, measurement of PGE_2, myeloperoxidase (MPO), various mRNAs, mucin as well as apoptosis and proliferation study, we used rectum and distal colon.

For labelling with BrdU to examine epithelial cell proliferation, 1 h before the mice were sacrificed, BrdU (100 mg/kg) was injected intraperitoneally as described previously (Kabashima et al., 2002).

The intestinal level of PGE_2 was determined by ELISA according to the manufacturer's protocol, as previously described (Futaki et al., 1993).

2.3. Myeloperoxidase (MPO) activity

MPO activity in the colonic tissues was measured as previously described (Krawisz et al., 1984; Tanaka et al., 2007). After 7 days of DSS treatment, animals were placed under deep ether anaesthesia and sacrificed. Colons were dissected, rinsed with cold saline and cut into small pieces. Samples were homogenized, freeze-thawed and centrifuged. The protein concentrations of the supernatants were determined using the Bradford method (Bradford, 1976). MPO activity was determined in 10 mM phosphate buffer with 0.5 mM o-dianidisine, 0.00005% (w/v) hydrogen peroxide and 20 μg protein. MPO activity was obtained from the slope of the reaction curve and its specific activity was expressed as the number of hydrogen peroxide molecules converted per min per mg protein.

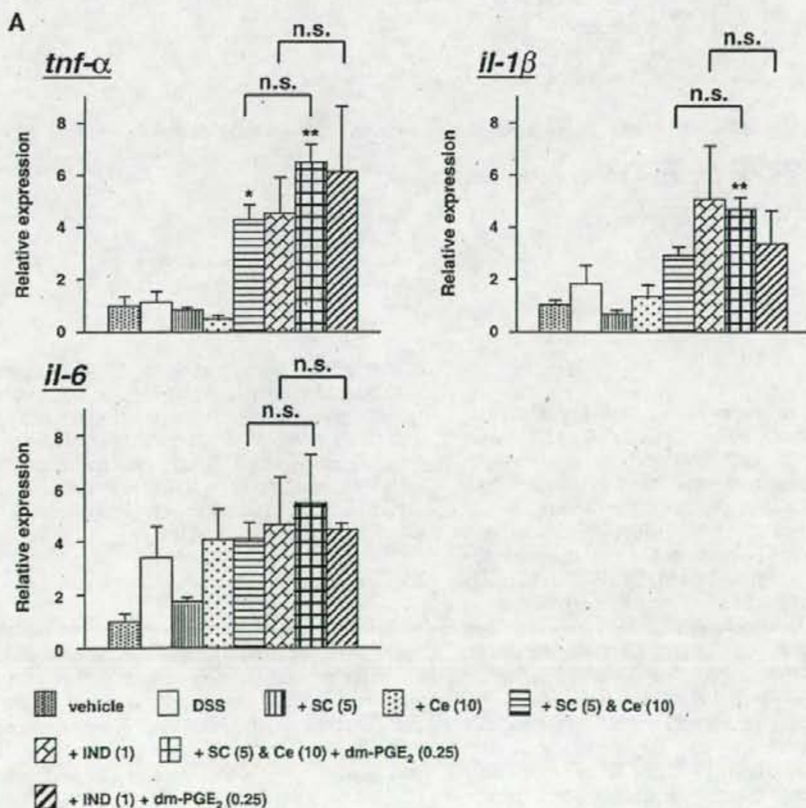

Fig. 4. Effect of NSAIDs and dm-PGE_2 on the mRNA expression of various proteins in the intestinal tissues of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. The relative mRNA expression of each gene in the intestinal tissues was monitored and expressed as described in the legend of Fig. 2A. Values are mean ± S.E.M. (n = 3–7). **(or ## or &&)$P < 0.01$; *(or # or &)$P < 0.05$; n.s., not significant.

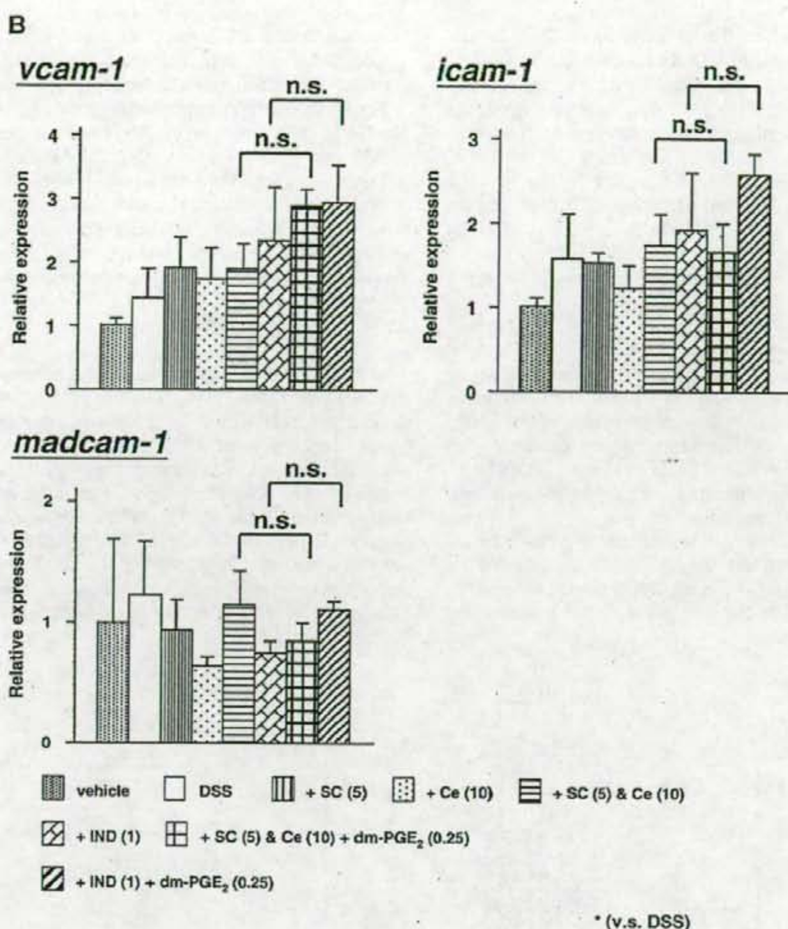

Fig. 4 (continued).

2.4. Real-time RT-PCR analysis

Total RNA was extracted from intestinal tissues using an RNeasy kit according to the manufacturer's protocol. Samples (2.5 µg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument (Bio-Rad)) experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software according to the manufacturer's instructions. The real-time PCR cycle conditions were 2 min at 50 °C, followed by 10 min at 90 °C and finally 45 cycles of 95 °C for 30 s and 63 °C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used were (name: forward primer, reverse primer): for mouse: *tumor necrosis factor (tnf)-α*: 5′-cgtcagccgatttgctatct-3′, 5′-cggactccgcaaagtctaag-3′; *interleukin (il)-1β*: 5′-gatcccaagcaatacccaaa-3′, 5′-ggggaactctgcagactcaa-3′; *il-6*: 5′-ctggagtcacagaaggagtgg-3′, 5′-ggtttgccgagtagatctcaa-3′; *vascular cell adhesion molecule (vcam)-1*: 5′-ctcctgcacttgtggaaatg-3′, 5′-tgtacgagccatccacagac-3′; *intercellular adhesion molecule (icam)-1*: 5′-tcgtgatggcagcctcttat-3′, 5′-gggcttgtcccttgagtttt-3′; *mucosal addressin cell adhesion molecule (madcam)-1*: 5′-gcaggctgggagctactct-3′, 5′-tccctcttgtggtaggttgc-3′; *cox-1*: 5′-cggtgacatcgatgctttag-3′, 5′-ggagcccccatctctatcat-3′; *cox-2*: 5′-tgctatctttggggagacca-3′, 5′-gctcggcttccagtattgag-3′; *muc2*: 5′-gctgacgagtggttggtgaatg-3′, 5′-gatgaggtggcagacaggagac-3′; *muc3*: 5′-cgtggtcaactgcgagaatgg-3′, 5′-cggctctatctctacgctctcc-3′; for rat; actin: 5′-gatcattgctcctcctgagc-3′, 5′-actcctgcttgctgatccac-3′; *muc2*: 5′-gaggacaggcccatctatga-3′, 5′-cagatcctccaggtgggtag-3′; *muc3*: 5′-atgcacaaaggcaagagtcc-3′, 5′-ctcaaagccaatgttgtgga-3′.

2.5. Histological and immunohistochemical analysis

Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned.

For immunohistochemical analysis, sections were treated in a microwave oven with 0.01 M citric acid buffer (pH 6.0) for antigen activation and incubated with 0.3% hydrogen peroxide-containing methanol for removal of endogenous peroxidase. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against BrdU (1:100 dilution) in the presence of 2.5% BSA and then incubated for 1 h with peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins (Envision kit). Then, 3, 3′-diaminobenzidine (DAB) was applied to the sections and the sections were finally incubated with Mayer's hematoxylin. Samples were mounted with malinol and inspected using a fluorescence microscope (Olympus BX51).

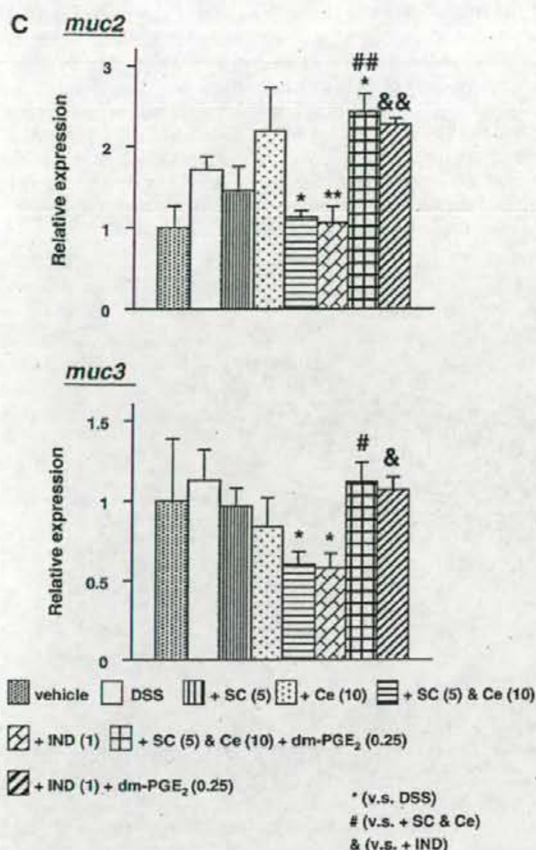

Fig. 4 (continued).

For staining of mucus with Alcian blue, sections were placed in 3% acetic acid for 3 min, then in 1% Alcian blue and 3% acetic acid (pH 2.5) for 10 min. Sections were then incubated in 0.1% nuclear fast red for 1 min as a counter stain and mounted with malinol.

2.6. TdT-mediated dUTP-biotin end labelling (TUNEL) assay

Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with TdTase and biotin 14-ATP for 1 h at 37 °C and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected using a fluorescence microscope (Olympus BX51).

2.7. Cell culture and staining with Hoechst 33342

Cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. Unless otherwise noted, cells (0.8×10^4 cells per well in 24-well plates, 4×10^4 cells per well in 6-well plates, 6×10^5 cells in 100-mm plates) were cultured for 24 h prior to use in experiments. Apoptotic chromatin condensation was monitored as described previously (Tsutsumi et al., 2002). Cells were washed with PBS, stained with 10 μg/ml Hoechst 33342 and observed under a fluorescence microscope.

2.8. Statistical analysis

All values are expressed as the mean ± standard error mean (S.E.M.). Two-way analysis of variance (ANOVA) followed by the Tukey test or the Student's t-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P<0.05$.

3. Results

3.1. Effect of various NSAIDs on DSS-induced colitis

The severity of DSS-induced colitis can be monitored by various indices, such as body weight, disease activity index and length of colon. We examined the effect of SC-560, a COX-1-selective inhibitor, on the time-course of development of colitis induced by 3% DSS administration by monitoring body weight and disease activity index. Administration of 3% DSS caused a mild increase in the (Fig. 1A), which is consistent with our previous results (Tanaka et al., 2007). Administration of SC-560 did not affect the DSS-induced increase in the disease activity index and alteration in body weight even at the highest tested dose (10 mg/kg) (Fig. 1A). Celecoxib, a COX-2-selective inhibitor did not worsen the DSS-induced colitis (Fig. 1B). It was reported that either SC-560 or celecoxib worsened the DSS-induced colitis (Okayama et al., 2007) and the discrepancy may be due to the difference in animal species and dose of DSS. Furthermore, disease activity index data showed weak amelioration of DSS-induced colitis by celecoxib (Fig. 1B). We also examined the effect of administration of a combination of SC-560 and celecoxib on development of DSS-induced colitis. As shown in Fig. 1C, administration of this combination to DSS-treated mice not only reduced body weight but also stimulated an increase in the disease activity index. We confirmed that in mice that had not been treated with DSS, administration of the SC-560/celecoxib combination did not affect body weight and the data not shown. Based on previous reports (Kato et al., 2001), the concentration of SC-560 or celecoxib employed in the experiments described in Fig. 1 C should have been sufficient to inhibit COX-1 or COX-2, respectively. Furthermore, administration of a non-selective NSAID (indomethacin) alone decreased the body weight and stimulated an increase in the disease activity index of DSS-treated mice to a similar extent as treatment with SC-560/celecoxib (Fig. 1C). The exacerbation of DSS-induced colitis by administration of SC-560/celecoxib or indomethacin was confirmed by monitoring another index of colitis, DSS-induced colon shortening, which is used as a morphometric measure for the degree of inflammation (Table 1). All of these results suggest that inhibition of both COX-1 and COX-2 exacerbates DSS-induced colitis.

In order to test this idea, we measured the intestinal level of PGE_2. At first we confirmed, by real-time PCR, that *cox-1* and *cox-2* mRNAs were expressed in the intestinal tissues (Fig. 2A). DSS-administration up-regulated the expression of *cox-2* mRNA but not of *cox-1* mRNA, as described previously (Fukata et al., 2006; Singer et al., 1998), however, the time-course profile was not consistent with data reported previously (Okayama et al., 2007). This may be due to the difference in animal species and dose of DSS. The results displayed in Fig. 2B show how the intestinal level of PGE_2 is altered with the development of DSS-induced colitis. The intestinal level of PGE_2 increased gradually for 5 days after initiation of DSS-treatment and then returned to the original level (Fig. 2B). This transient profile was not observed in previous report and again this may be due to the difference in animal species and dose of DSS. Fig. 2C shows the effect of administration of various NSAIDs on the intestinal level of PGE_2 in DSS-treated mice. Administration of SC-560 weakly decreased the intestinal level of PGE_2; however, celecoxib did not affect the level significantly (Fig. 2C). Furthermore, administration of SC-560/celecoxib dramatically decreased the intestinal level of PGE_2 to a similar extent as was observed in response to indomethacin administration (Fig. 2C). Combining the results summarised in Table 1, Figs. 1 and 2, it seems

that the observed large decrease in the intestinal level of PGE_2 is required for the exacerbation of DSS-induced colitis by NSAIDs.

3.2. Effect of PGE_2 on the exacerbation of DSS-induced colitis by NSAIDs

To confirm the role of PGE_2 in exacerbation of DSS-induced colitis by NSAIDs, we have examined the effect of exogenously administered PGE_2 in this disease model. As shown in Fig. 3A, administration of dm-PGE_2 (a stable analogue of PGE_2) returned the body weight and disease activity index of SC-560/celecoxib- or indomethacin-administered DSS-treated mice to a similar level to that observed in control mice (without NSAID-administration). Similar results were obtained with another index of colitis, colon length (Fig. 3B). Colonic MPO activity, an indicator of leukocyte infiltration, was also increased by administration of SC-560/celecoxib or indomethacin and this increase was suppressed by simultaneous administration of dm-PGE_2 (Fig. 3C). The results shown in Fig. 3 show that administration of dm-PGE_2 suppresses the exacerbation of DSS-induced colitis by NSAIDs and suggest that the decrease in the intestinal level of PGE_2 is responsible for the exacerbation of DSS-induced colitis by NSAIDs.

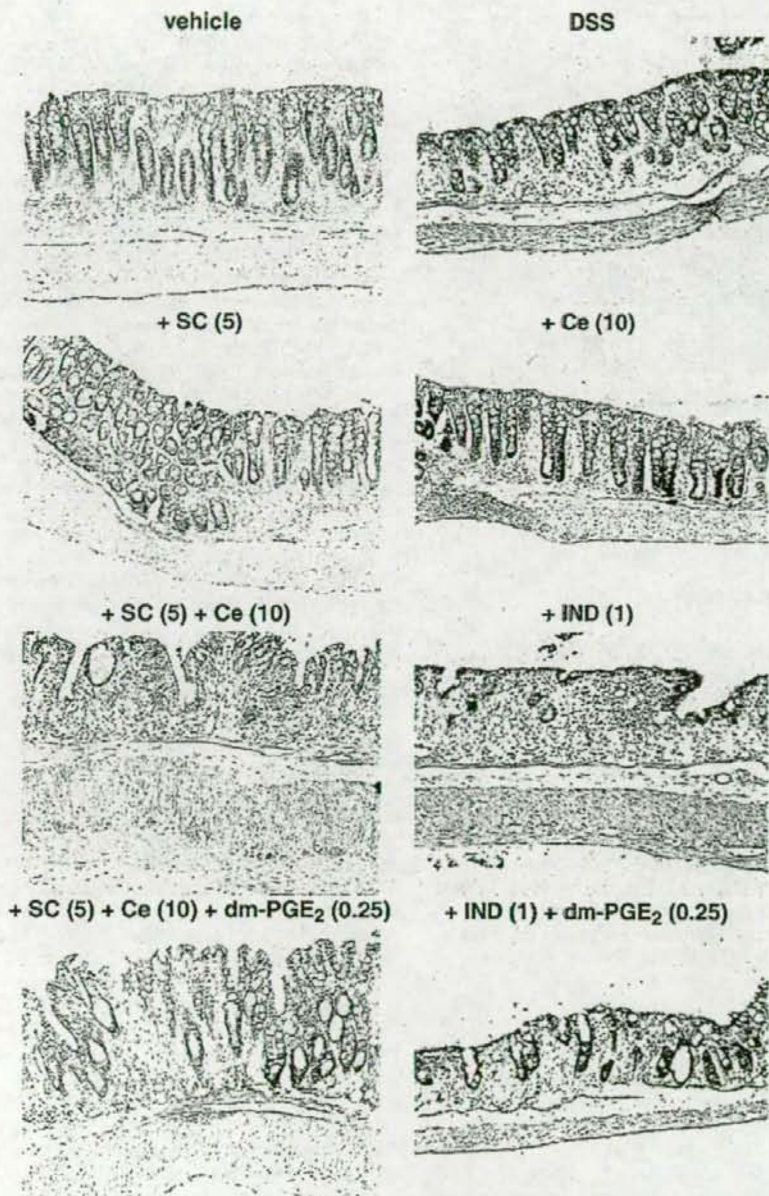

Fig. 5. Effect of NSAIDs and dm-PGE_2 on the amount of mucus in the intestinal mucosa of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. Sections of colonic tissues were prepared and subjected to staining with Alcian blue as described in the Materials and methods. Magnification of all photomicrographs is ×100.

3.3. Involvement of cytokines, cell adhesion molecules and mucin proteins in exacerbation of DSS-induced colitis by NSAIDs

Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 and cell adhesion molecules, such as ICAM-1, VCAM-1 and MAdCAM-1, play an important role in the activation and infiltration of leukocytes that is associated with inflammatory bowel disease. In order to understand the mechanism governing the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2, we compared the mRNA expression of various inflammatory bowel disease-related proteins, such as cytokines, cell adhesion molecules and mucin proteins in the intestinal tissues of DSS-treated mice. As shown in Fig. 4A, *tnf-α* mRNA expression was induced by administration of SC-560/celecoxib; however, this induction was not suppressed by simultaneous administration of dm-PGE_2. Neither SC-560/celecoxib nor dm-PGE_2 affected *il-1β* or *il-6* mRNA expression (Fig. 4A). Likewise, *vcam-1*, *icam-1* and *madcam-1* mRNA expression was not affected by SC-560/celecoxib or dm-PGE_2 (Fig. 4B). We previously reported that DSS-treatment up-regulated the mRNA expression of these cytokines and cell adhesion molecules (Tanaka et al., 2007). The ineffectiveness of DSS on expressions of these factors may be due to that we measured 3 days (instead of 7 days in our previous paper) after the initiation of DSS treatment. On the other hand, *muc2* and *muc3* mRNA expression was inhibited by administration of SC-560/celecoxib or indomethacin, and simultaneous administration of dm-PGE_2 counteracted this effect (Fig. 4C). Staining of tissue with Alcian blue demonstrated that the administration of indomethacin or SC-560/celecoxib decreased the amount of mucus (blue-staining spots) in the intestinal mucosa of DSS-treated mice. Simultaneous administration of dm-PGE_2 counteracted this effect, with mucin levels in these mice equivalent to that of controls (Fig. 5). The results displayed in Figs. 4 and 5 suggest that expression of mucin proteins rather than expression of cytokines and cell adhesion molecules is involved in the observed exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2.

We also examined the effect of NSAIDs and PGE_2 on the mRNA expression of cytokines, cell adhesion molecules and mucin proteins *in vitro*. In RAW264 cells, indomethacin did not affect *tnf-α*, *il-1β* and *il-6* mRNA expression (Table 2A). Similarly, in bEnd.3 cells, indomethacin did not affect *vcam-1*, *icam-1* and *madcam-1* mRNA expression (Table 2B). We confirmed that treatment of these cells with LPS up-regulated the mRNA expression of these genes (up-regulation of *tnf-α* mRNA expression was observed 3 h (data not shown) but not 24 h (Table 2A) after the addition of LPS), which is consistent with previous results (Gupta et al., 1995). On the other hand, in IEC6 cells, *muc2* and *muc3* mRNA expression was inhibited by indomethacin (Table 2C). Treatment of cells with dm-PGE_2 stimulated mRNA expression of these genes (Table 2C), suggesting that indomethacin directly inhibits mRNA expression of mucin proteins through decreasing the level of PGE_2. This effect may be responsible for the decrease in the expression of mucin proteins observed after the administration of indomethacin or SC-560/celecoxib in DSS-treated mice (Figs. 4 and 5).

3.4. Involvement of intestinal mucosal apoptosis and cell growth in the exacerbation of DSS-induced colitis by NSAIDs

Inhibition of cell growth and induction of cell death at the intestinal mucosa also play an important role in the pathogenesis of inflammatory bowel disease, because the intestinal mucosa functions as a barrier against pathogenic bacterial invasion. Inhibition of cell growth and induction of apoptosis at the intestinal mucosa was reported in both inflammatory bowel disease patients and animal models of inflammatory bowel disease (Kabashima et al., 2002; Souza et al., 2005). The cell death associated with inflammatory bowel disease seems to be induced mainly by reactive oxygen species. Mucin proteins (such as MUC2 and MUC3) also function as a barrier to bacterial invasion and mucosal damage by reactive oxygen species (Van der Sluis et al., 2006). Therefore we examined, using the TUNEL assay, the effect of indomethacin administration on the level of apoptosis observed in the intestinal mucosa of DSS-treated mice. More TUNEL-positive cells (apoptotic cells) were observed in the intestinal mucosa of the indomethacin- or SC-560/celecoxib-administered mice than the control mice (Fig. 6A). Furthermore, simultaneous administration of dm-PGE_2 counteracted this effect, with the extent of apoptosis in those animals similar to that of controls (Fig. 6A).

Table 2
Effect of indomethacin and dm-PGE_2 on mRNA expression of various proteins in vitro

A

Treatment	Relative expression		
	tnf-α	*il-1β*	*il-6*
Control	1.00±0.09	1.00±0.08	1.00±0.09
Indomethacin (50 μM)	0.94±0.06	1.28±0.05	1.12±0.06
Indomethacin (100 μM)	0.90±0.05	1.26±0.07	0.91±0.09
LPS	1.11±0.08	3.00±0.25[b]	110±6.40[b]

B

Treatment	Relative expression		
	vcam-1	*icam-1*	*madcam-1*
Control	1.00±0.02	1.00±0.05	1.00±0.09
Indomethacin (50 μM)	0.98±0.07	1.15±0.06	1.12±0.06
Indomethacin (100 μM)	0.91±0.06	1.02±0.07	0.91±0.09
LPS	11.1±0.37[b]	4.94±0.37[b]	44.6±3.54[b]

C

Treatment	Relative expression	
	muc-2	*muc-3*
Control	1.00±0.09	1.00±0.07
Indomethacin (100 μM)	0.49±0.12[b]	0.24±0.01[a]
Indomethacin (100 μM)+ dm-PGE_2 (0.5 μM)	2.00±0.13[b,c]	5.05±0.13[a,c]

RAW264 (A) or bEnd.3 (B) cells were incubated with the indicated concentrations of indomethacin (or 5 μg/ml LPS) for 24 h or 18 h, respectively (A, B). IEC6 cells were pre-incubated with or without 0.5 μM dm-PGE_2 for 1 h and further incubated with the indicated concentrations of indomethacin for 24 h in the presence of the same concentration of dm-PGE_2 as was used in the pre-incubation step (C). Relative mRNA expression of each gene was monitored and expressed as described in the legend of Fig. 2A. Values shown are mean±S.E.M. ($n=3$).
[a] $P<0.05$, vs. Control; [b] $P<0.01$, vs. Control; [c] $P<0.01$, vs. Indomethacin (100 μM).

We also examined, using a BrdU-staining method, the effect of indomethacin administration on intestinal mucosal cell proliferation in DSS-treated mice. Less BrdU-positive cells (growing cells, cells stained brown) were observed at the intestinal mucosa of indomethacin- or SC-560/celecoxib-administered mice than the control mice (Fig. 6B, C). Furthermore, simultaneous administration of dm-PGE_2 with the indomethacin resulted in similar numbers of BrdU-positive cells to the control (Fig. 6B, C). The results shown in Fig. 6 suggest that alterations to the levels of cell proliferation and apoptosis in intestinal mucosa are involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2.

We also examined the effect of indomethacin and PGE_2 on apoptosis and cell growth *in vitro*. As shown in Fig. 7A, the relative number of IEC6 cells with apoptotic chromatin condensation was not increased by treatment with indomethacin, showing that indomethacin did not induce apoptosis under these experimental conditions.

We also used menadione, a superoxide anion (a representative reactive oxygen species) releasing drug, to examine the effect of PGE_2 on reactive oxygen species-induced cell death. As shown in Fig. 7B, cell death induced by treatment with menadione for 24 h was partially suppressed by simultaneous treatment of cells with 0.5 μM dm-PGE_2. We concluded that the cell death described in Fig. 7B was mediated by apoptosis, based on monitoring apoptotic chromatin condensation (data not shown). These results show that PGE_2 protects intestinal cells from reactive oxygen species-induced apoptosis.

Indomethacin significantly inhibited the growth of IEC6 cells and this growth inhibition was suppressed by simultaneous treatment of cells with dm-PGE_2 (Fig. 7C). This suggests that indomethacin directly

inhibited the growth of the intestinal cells through decreasing the level of PGE_2 and this effect may be responsible for the inhibition of epithelial cell proliferation in the intestine of DSS-treated mice after the administration of indomethacin *in vivo* (Fig. 6B and C).

4. Discussion

In both clinical and animal studies, it is debatable as to whether NSAIDs are of benefit, have no effect or aggravate inflammatory bowel disease and inflammatory bowel disease-related experimental colitis (Bonner, 2001; Bonner et al., 2000; El Miedany et al., 2006; Evans et al., 1997; Felder et al., 2000; Mahadevan et al., 2002; Takeuchi et al., 2006; Yamada et al., 1993). To utilize NSAIDs for the clinical treatment of inflammatory bowel disease but avoid exacerbation of inflammatory bowel disease by NSAIDs, it is important to know what types of NSAIDs ameliorate or exacerbate the development of inflammatory bowel disease and to understand the underlying molecular mechanisms. In this study, focusing on COX-1/COX-2 specificity, we have examined the effect of various NSAIDs on DSS-induced colitis. Administration of either a COX-1-selective inhibitor (SC-560) or of a COX-2-selective inhibitor (celecoxib) did not affect DSS-induced colitis, however, the colitis was clearly exacerbated by the administration of these drugs in combination or administration of the non-selective NSAID indomethacin. For the combination experiment (the results of which are shown in Fig. 1 and Table 1),

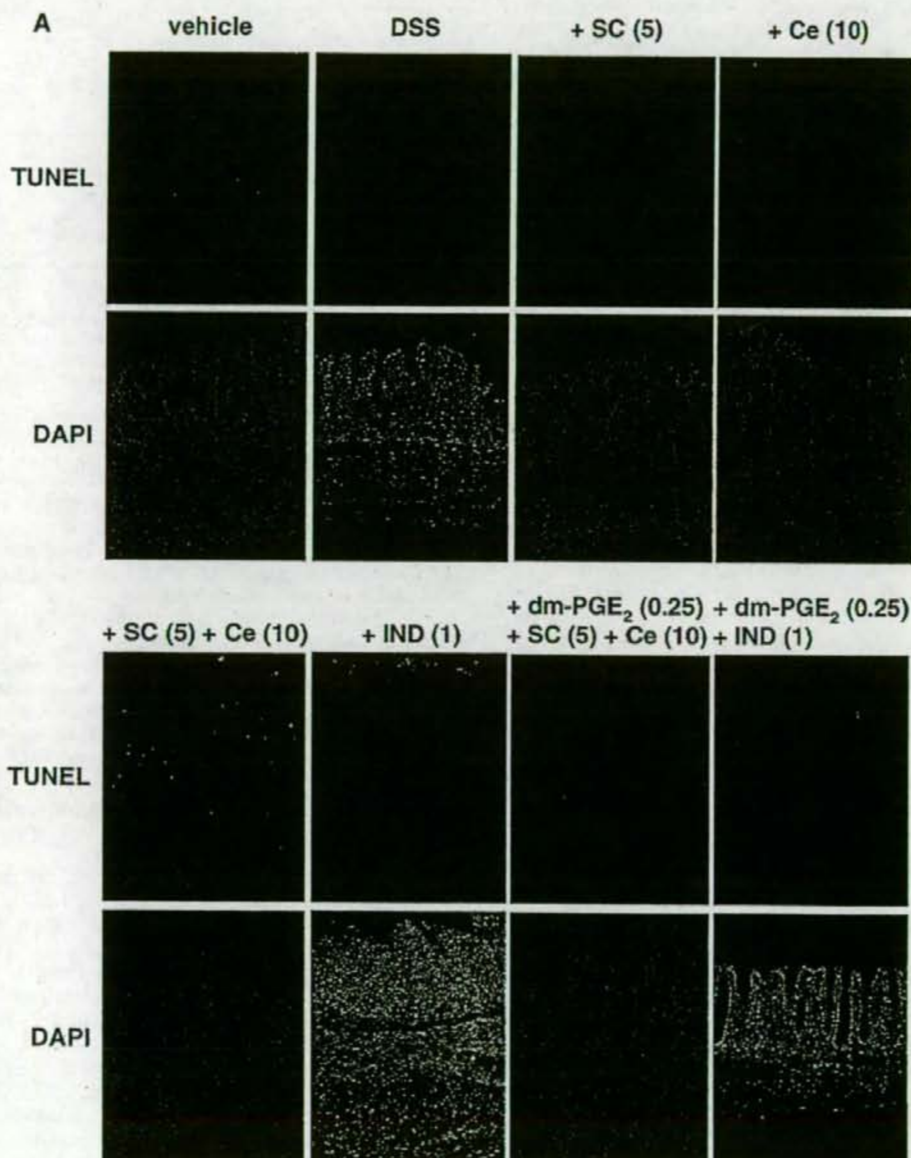

Fig. 6. Effect of NSAIDs and dm-PGE_2 on the level of apoptosis and cell proliferation in the intestinal mucosa of DSS-treated mice. DSS-induced colitis was developed and NSAIDs and dm-PGE_2 were administered as described in the legend of Fig. 3. Sections of intestinal tissues were prepared and subjected to the TUNEL assay and DAPI staining (A) or to immunohistochemical analysis with an antibody against BrdU (B). Cells (more than 400 cells) were counted for staining with BrdU in four independent sections. Values shown are mean±S.E.M. **(or ## or &&)$P<0.01$ (C). Magnification of all photomicrographs is ×100 (A) or ×200 (B).